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ORIGINAL ARTICLE

Labeling of nucleosides with fluorescamine and detection by spectrofluorometer for End Stage Renal Disease

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Ultrasonic assisted microextraction

Abstract Nucleosides are characterized as biomarkers in AIDS, Alzheimer, tumor, breast cancer and various malignant diseases. In the present work a direct method for the detection of nucleosides (adenosine, cytidine, uridine and guanosine) from urine samples has been developed. Nucleosides represent the extent of damage in genetic material, analysis of nucleosides by ultrasonic assisted microextraction effectively eliminates the interfering constituent of urine. This has made it a highly selective and sensitive method to analyze the nucleosides with a lower limit of detection 0.220 μmol/L and Limit of quantitation 0.660 μmol/L. The method has been validated with good linearity and correlation of coefficients of the calibration curves was higher than 0.997. The coefficients were in the range of 0.11–16.92% (inter-day) and 0.38–16.43% (intra-day), respectively.

1. Introduction

Urinary proteome analysis is an emerging field of proteomics in research. Nucleosides are important constituents of RNA which can be metabolized and reutilized by nucleic acid. Subunits of nucleic acid are nucleosides as they consist of a nucleobase bound to pentose sugar, and modified in various ways during metabolism. It has been reported that liquid chromatography, LC/MS, GC/MS (Ravanat et al., 1999; Pouget et al., 2000; Evans et al., 1999) and HPLC are more sensitive and specific for the analysis of nucleosides, although these methods require more complex steps and time for extraction of nucleosides from the urine. In the cases of end stage renal disease and renal failure these nucleosides do not degrade but excreted in the urine because of lack of specific phosphorylase and serve as potential markers (Gehrke et al., 1979; Rasmuson and Bjork, 1995).Commonly used method for urinary nucleosides are HPLC/MS, LCMS, LC/ESI-MS etc. with conventional detectors. A statistical survey states that excretion of nucleosides and modified nucleosides in the urine correlates with the severe malignancy of renal failure and cancer in patients (Speer et al., 1979; Nakano et al., 1993).

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An important class of derivatization is attachment of fluorophore in the protein by a complex formation and change in the structure. The result may change in fluorescent intensity, emission or excitation and this measurement provides information of the molecule excreted in the biological fluid. Cytidine, uridine, adenosine and guanosine are four nucleosides which are secreted and elevated during breast cancer, end stage renal diseases kidney failure etc. (Hsu et al., 2011; Jiang and Ma, 2009). It is known that free radicals in the body react with biomolecules and involve in damaging and lead to several diseases through inducing kidney failure, neurodegenerative diseases and cardiac failure (Zambonin et al., 1999; Haegele et al., 1998). The present method investigates the four potential nucleosides which help in determining different stages of patients suffering from end stage renal disease without the usage of any ion pair reagent and bulky instrument.

2. Materials and methods

2.1. Chemicals

All chemicals were prepared with Mili Q system (Milipore, Bedford, MA, USA).The fluorescamine is of analytical purity

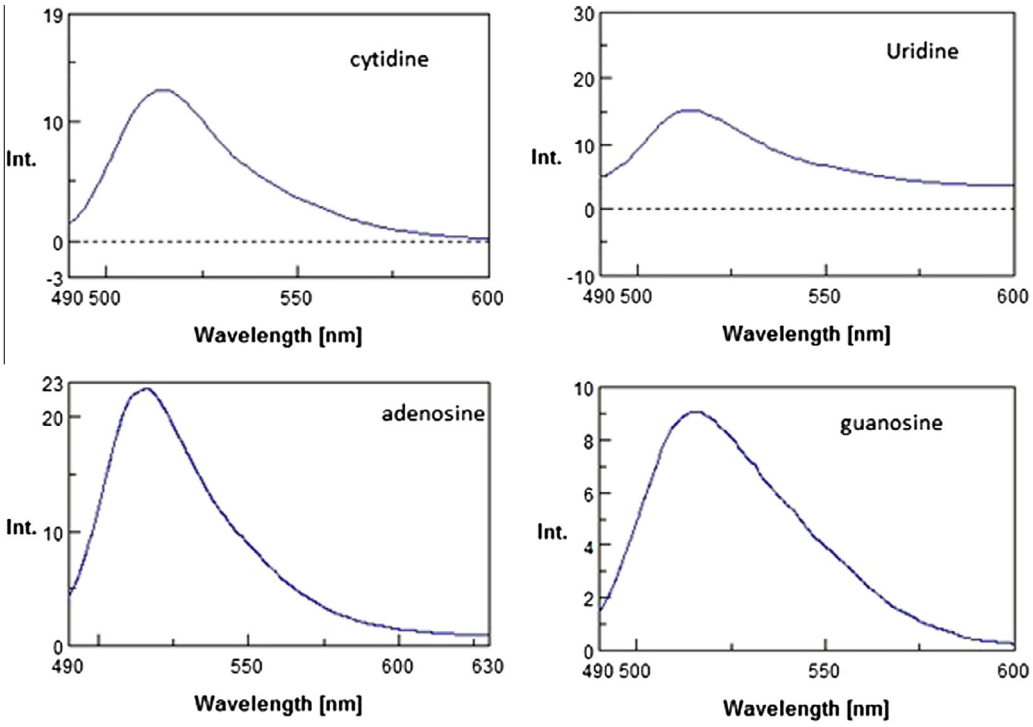

Figure 1 Spectra of four spiked nucleosides in patients' urine at a concentration of 10 μg/L in distilled water, pH = 5 sodium acetate buffer. Excitation wavelength for the fluorescence was 490–530 nm.

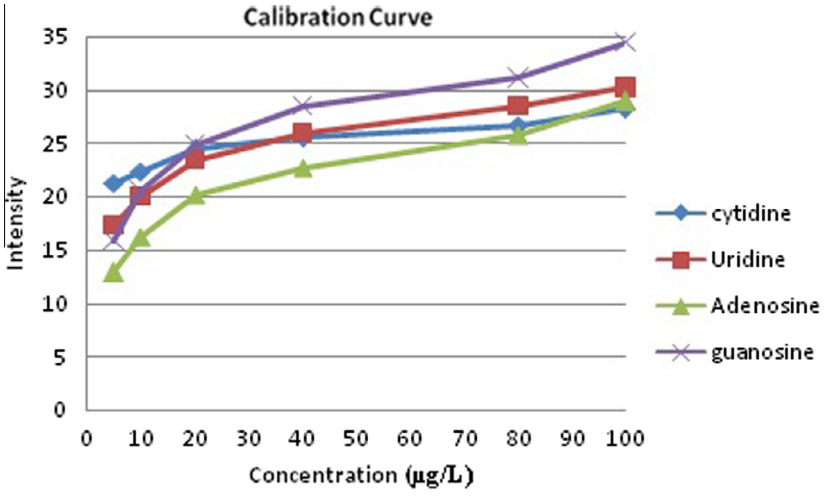

Figure 2 Effect of concentration on absorption and fluorescence spectra of nucleosides.

Table 1 Linearity (as coefficient correlation R^2), slope, limit of detection, limit of quantification in different concentration ranges for four nucleosides in biological matrices).

Nucleosides	Urine				
	Concentration range (μg/L) $y = a + bx$[a]	R^2	Slope	LOD[b] μg/L	LOQ[c] μg/L
Cytidine	1.616	0.991	1.384	1.015	3.335
Uridine	1.121	0.993	2.643	1.021	3.31
Adenosine	2.53	0.997	3.177	0.452	1.624
Guanosine	1.41	0.99	3.648	0.221	0.6606

[a] x = concentration, μg/L; y = peak area; a = intercept; b = slope; calibration graph constructed over six concentration levels; results are the averages of three replicate analyses.
[b] LOD = limit of detection (S/N = 3), μg/L.
[c] LOQ = limit of quantitation (S/N = 10) μg/L.

Table 2 Intraday and inter day precision of four nucleosides.

Nucleosides	Intra day			Intra day		
	Amount added (μg/L)	Amount found (Average)	R.S.D (CV %)	Amount added (μg/L)	Amount found (Average)	R.S.D (CV %)
Cytidine	10	22.21	1.48	10	20.099	2.23
	40	24.72		40	24.38	
	100	28.62		100	27.96	
Uridine	10	19.45	1.91	10	19.83	4.41
	40	25.37		40	25.92	
	100	30.24		100	30.15	
Adenosine	10	16.43	0.37	10	16.16	3.14
	40	22.17		40	22.63	
	100	28.37		100	28.44	
Guanosine	10	20.08	2.59	10	20.27	1.21
	40	28.14		40	28.24	
	100	33.35		100	34.24	

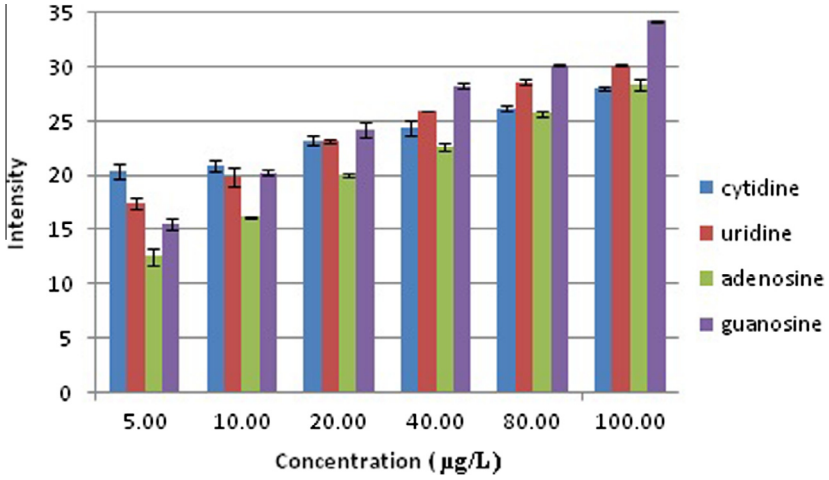

Figure 3 Representing standard deviation and average mean difference of four nucleosides at different concentration, pH = 5.

and was purchased from Aldrich (Aldrich France). Four nucleoside standards i.e., cytidine (purity 99%), uridine (purity 99%), adenosine (purity 99%), and guanosine (purity 99%) were provided from Sisco Research Laboratory Pvt. Ltd.

2.2. Preparation of standard nucleosides solution

Urine samples from patients were collected from the Kidney Hospital (Ahmedabad, Gujarat).The samples were stored at

Table 3 Represents the average recovery ranged 95.73–104.7%.

Nucleosides	Recovery (%)			
	10 μg/L		40 μg/L	
	Average ± SD	RSD (%)	Average ± SD	RSD (%)
Cytidine	104.75 ± 0.77	1.48	101.50 ± 0.94	1.56
Uridine	97.72 ± 0.62	1.91	96.11 ± 0.40	0.12
Adenosine	95.73 ± 0.38	0.33	80.16 ± 0.72	2.18
Guanosine	99.45 ± 0.42	2.59	89.61 ± 0.48	0.80

−80 °C immediately after collection. We used pooled urine from four normal female (healthy, patient) subjects. The patients were not treated with any drugs or chemicals before the urine was collected. The frozen urine samples were thawed and kept at room temperature for 5 min. A 500-μL aliquot of urine was pipetted into a 1.5 mL centrifuged tube purchased from Supelco (Bellefonte PA), capped, and then centrifuged for 5 min at 15,000 rpm. The supernatant was filtered through a filter (Millipore). 1 mL of supernatant was collected and derivatized with fluorescamine with sodium acetate buffer for an analysis of urinary nucleosides. 10 μg/L stock solution of nucleosides was prepared in distilled water and kept at −20 °C. The working solutions were prepared in a Mili Q system (Milipore, Bedford, MA, USA) by diluting stock solution to the concentration range of 5–100 μg/L (5, 10, 20, 40, 80, and 100 μg/L).

2.3. Fluorescence derivatization

100 μl of supernatant potion of standard analyte was mixed with 850 μl of sodium acetate buffer and 50 μl of fluorescene. Then, mixture liquid was incubated for 2–6 min simultaneously and the final solution analyzed by a spectrofluorometer (JASCO FP-6500).

3. Results and discussion

Identification and quantification of nucleosides were determined by comparing the standard nucleoside solution with spiked urine sample. Using our method, we can detect four nucleosides in the urine of patients without tedious extraction process and act as potential biomarkers for determination of end stage renal diseases as shown in Fig. 1.

3.1. Linearity

Calibration curves were plotted between the concentration 1×10^{-9}–1×10^{3} M of four nucleosides and found to be rectilinear over the range of 10–100 μmol/L as shown in Fig. 2. The correlation coefficient (R^2) was higher than 0.987, limit of detection (LOD) and limits of quantification (LOQ) are given in Table 1.

3.2. Precision

The precision of analytical method was determined by comparing the retention time of nucleoside standard and was determined in triplicate for validation. The coefficient of variation CV% within a day was in the range of 0.37–2.59%. Table 2 represents interday and intraday repeatability in terms of percentage RSD in retention time and peak area as shown in Fig. 3. Inter day repeatability was measured for 30 days as shown in Table 2. The data satisfied the criteria of 15% (CV) for both within day and intraday validation.

3.3. Recovery

The extraction recoveries were determined by comparing the corrected peak areas of nucleosides extracted from spiked urine samples with those of unextracted standard containing the same amount of nucleosides. For the determination of nucleosides in the urine three replicate analyses of samples spiked at the concentration of 5 and 10 μmol/mL were carried out. The same procedure for the sample preparation and derivatization as described in the previous section is shown in Table 3.

4. Conclusion

Our method provides a direct measurement of nucleosides, compared to other detection methods. This process has its ability to find early diagnostic markers because this biological specimen is not easy to collect, analyze and requires many complex steps to extract. The absorption of fluorescence spectra decides the constant release of nucleosides from the urine in kidney patients as early diagnostic markers in ESRD. The validated method was shown to have sufficient selectivity and sensitivity to quantify the selected nucleosides in the human urine. The developed method was simple, selective, and reproducible with good recovery. The linearity and reproducibility were also acceptable in the linear range from 0.2–400 μmol/L.(Lee et al., 2004) Therefore, our area of potential study states that damage and repair of biomolecules leads to diseased condition. We conclude that this method is reliable in assessing the urinary profiles of these nucleosides for diagnosis of several diseases.

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